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Role of IS*1* in the conversion of virulence (Vi) antigen expression in Enterobacteriaceae

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Summary. When *Escherichia coli* HB101 harbors pWR127, a plasmid comprising the *viaB* gene from *Citrobacter freundii* WR7004 and the ColE1-derived pACKC1, the strain produces the virulence (Vi) antigen. Vi antigen expression is abolished (Vi^- phenotype), however, when an IS*1* or IS*1*-like DNA element inserts into the *viaB* region. To determine the sites of IS*1* insertion, pWR127 DNAs extracted from 95 independently isolated Vi^- strains were analyzed by digestion with the restriction endonuclease *Pst*I and agarose gel electrophoresis. Ten insertion sites were found distributed nonrandomly in an area of about 1.3 kb. Nine Vi^+ strains (two *Citrobacter*, two *E. coli*, and five *Salmonella* strains), four of which contain pWR127, were then tested for the presence of IS*1* by DNA-DNA hybridization. Of the nine strains, five were stable Vi^+ and did not contain IS*1*. The other four which generated Vi^- strains, contained IS*1*. When pRR134, a plasmid that contains IS*1* was transferred into a stable Vi^+ *Salmonella typhimurium* strain carrying pWR127 (OU5140), Vi^- strains were produced from which pWR127 derivatives carrying IS*1* inserts could be isolated. It appears, therefore, that the presence of an IS*1* or IS*1*-like element in a strain is required for conversion of the Vi^+ expression state to the Vi^- expression state.

Key words: Vi antigen – IS*1* – Insertion – Gene expression – DNA-DNA hybridization

Introduction

The virulence (Vi) antigen (Felix et al. 1934; Snellings et al. 1981; Baron et al. 1982) is a capsular molecule consisting of a homopolymer of galactosaminuronic acid and is presumably a virulence factor for *Salmonella typhi*. Its expression is controlled by two well separated chromosomal genes, *viaA* and *viaB;* the former is probably a regulatory gene (Johnson et al. 1965, 1966; Ou et al. 1988; Snellings et al. 1981) and has recently been shown to be identical to *rcsB* (Houng et al. 1991; see Stout and Gottesman 1990, for *rcsB*). Some strains of *Citrobacter freundii* also produce the Vi antigen; unlike *S. typhi*, however, its expression in *C. freundii* is unstable: cells oscillate between the expression (Vi^+) and non-expression (Vi^-) states with a high frequency (Snellings et al. 1981; Ou et al. 1988). To investigate this phenomenon, recombinant plasmid pWR127, which contains the *viaB* region derived from *C. freundii* strain WR7004, was isolated (Rubin et al. 1985). When pWR127 is introduced into *Escherichia coli* HB101, which contains *viaA* but not *viaB*, the Vi antigen is readily expressed. Conversion from Vi^+ to Vi^- is also observed in this strain with high frequency, but the reverse, i.e. switching from Vi^- to Vi^+, has never been observed (Ou et al. 1988). IS*1* causes mutations by inserting into genes (Fiandt et al. 1972; Hirsch et al. 1972). Various analyses of pWR127 samples obtained from Vi^+ and Vi^- strains have demonstrated that insertion of an IS*1*-like DNA element into the *viaB* region is the cause of the Vi^+ to Vi^- conversion in pWR127-bearing *E. coli* HB101 cells (Ou et al. 1988). This finding suggests that reversion to the Vi^+ state, as in the *C. freundii* strain WR7004, requires that the IS*1* element be excised. *C. freundii*, however, has been reported to be devoid of IS*1* (Nyman et al. 1981).

To define the role of the IS*1*-like element in this switching phenomenon, we have undertaken a series of experiments to enumerate the IS*1* insertion sites and to determine the relationship between the ability to generate Vi^- strains and the presence of IS*1* in various strains. Our results indicate that there are many IS*1* insertion sites in *viaB*, which, however, are confined to a particular region of the gene, and that for insertion into *viaB* to occur, the host bacterium must carry IS*1* or an IS*1*-like DNA element.

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Materials and methods

Bacterial strains and plasmids. Table 1 lists the properties of bacterial strains and plasmids used; other strains are described in the text. Clinical isolates were collected from the Pathology Laboratory of Chang Gung Memorial Hospital, Linkow, Taiwan. The plasmid pWR127 is a recombinant plasmid consisting of pACKC1, a ColE1-derived vector, and the 18 kb *viaB* region derived from *C. freundii* WR7004 (Rubin et al. 1985). Plasmid pRR134, a recombinant plasmid consisting of pBR322 and a DNA fragment containing IS*1* (Peterson et al. 1982), was a gift from R. Rownd.

Media and antibiotics. Media used were meat extract agar for agar plates and Pennassay broth (Difco) for liquid culture. Kanamycin (25 μg/ml) was added as needed.

DNA extraction, restriction endonucleases, and agarose gel electrophoresis. Plasmid and chromosomal DNA isolation was carried out as described (Ou et al. 1988). Restriction endonucleases *Alu*I, *Eco*RI, and *Pst*I were used according to the specifications of the manufacturer (Bethesda Research Laboratories). In general, 0.7% or 1% agarose gels were routinely used for electrophoresis (Ou et al. 1988).

Transformation and identification of Vi strains. Transformation was carried out using the method previously described (Ou et al. 1986). To identify Vi^+ and Vi^- colonies, the method described earlier (Ou et al. 1988) was employed. Briefly, colonies were examined with a stereoscopic microscope using oblique illumination from below the specimen stage (Landy 1950). With such illumination, Vi^+ colonies showed an orange hue, while Vi^- colonies were dull gray in colour. When necessary, the Vi^+ or Vi^- phenotype was confirmed by slide agglutination tests using anti-Vi serum (Difco).

DNA-DNA Hybridization. Denatured target DNA was spotted directly onto membrane filters and hybridized with labelled probe as described (Southern 1975; Ou et al. 1988). *In situ* hybridization to colonies was performed essentially as described by Rubin et al. (1985). As a probe, the 496 bp *Alu*I fragment of IS*1* (Ohtsubo and Ohtsubo 1978) was used. This fragment was recovered from agarose after electrophoresis of *Alu*I-digested pRR134 DNA. The radioactive probe was prepared by the random primer method (Feinberg and Vogelstein 1983, 1984).

Results

Insertion hotspots for IS1-like elements

We previously reported (Ou et al. 1988) the presence of an insertional hot spot for an IS*1*-like DNA element (for simplicity, the term IS*1* will be used throughout) in pWR127. This was based on the analysis of fragment profiles generated by *Pst*I from a number of Vi^- strains derived from *E. coli* HB101/pWR127. Most of these were found to carry IS*1* inserted into pWR127 in the same orientation and at the same site. Sporadically, a few strains were shown to produce a different profile, which, however, was due to IS*1* insertion in the other orientation

Table 1. Bacterial strains and plasmids

	Strain	Species[a]	Phenotypic description	Source[b]
1	WR7004	C.f.	The original strain that reversibly expresses the Vi antigen	WRAIR
2	OU7177	C.f.	A clinical isolate; reversibly expresses the Vi antigen	This laboratory
3	OU5140	S.t.	OU5027 carrying pWR127; OU5027 is a Fisher strain cured of the virulence plasmid; pWR127 is a recombinant plasmid containing the *viaB* region	This laboratory
4	OU5146	S.t.	OU5058 carrying pWR127; OU5058 is an LT2 strain cured of the virulence plasmid	This laboratory
5	OU5316	S.t.	OU5048 carrying pWR127; OU5048 is a C5 strain cured of the virulence plasmid	This laboratory
6	OU6060	*S. typhi*	*viaB*$^+$ was transferred from a C.f. Hfr strain by mating	WRAIR
7	OU7178	*S. dublin*	*viaB*$^+$	WRAIR
8	KP246	E.c.K-12	Carries pRR134, a derivative of pBR322 carrying a fragment containing IS*1*	R. Rownd[c]
9	OU3210	E.c.K-12	Strain HB101 harboring pWR127	This laboratory
10	OU3327	E.c. C	An *E. coli* C strain carrying the *viaB* region of WR7004	WRAIR

[a] C.f., *Citrobacter freundii*; S.t., *Salmonella typhimurium*; E.c., *Escherichia coli*
[b] WRAIR, Walter Reed Army Institute of Research, Washington, D.C., USA
[c] See Peterson et al. (1982)

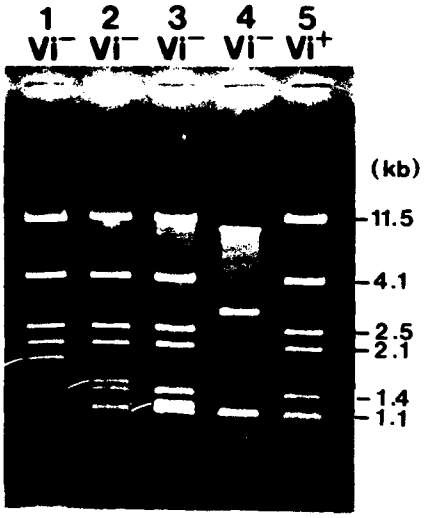

Fig. 1. Electrophoretic fragment profiles of Vi⁺ or Vi⁻ pWR127. Purified Vi⁺ or Vi⁻ pWR127 DNAs were treated with restriction endonuclease *Pst*I and electrophoresed in a 1% agarose slab gel. Lanes 1, 2, and 3, independent isolates of Vi⁻ pWR127; lane 4, Vi⁻ pWR127 showing a deletion; lane 5, Vi⁺ pWR127. *Arrowheads* indicate additional fragments due to IS*1* insertion into the largest fragment. The different sizes reflect different insertion sites: lane 1, at 1.75 kb; lane 2, at 1.5 kb; and lane 3, at 1.22 kb from the junction with the vector. The molecular weight of each fragment generated from Vi⁺ pWR127 by *Pst*I is indicated on the left

at the same site. Later, we found profiles that could not be explained simply by differences in insertion orientation, suggesting that more than one insertion site is used. We therefore carried out a quantitative analysis to examine the distribution of IS*1* insertions in the *viaB* region of pWR127. Each Vi⁺ *E. coli* HB101/pWR127 colony was transferred to liquid medium, grown and then plated for single colonies. Two Vi⁻ strains were randomly picked from each plate. Their plasmids were isolated and restriction fragment profiles generated by digestion with *Pst*I, which cleaves the IS*1* element at a single site (Ohtsubo and Ohtsubo 1978), were analyzed to locate the insertion site (Ou et al. 1988). Three types of restriction profiles were found (see Fig. 1): one is identical to that of the Vi⁺ control, one indicates an insertion of IS*1*; and the third shows an apparent deletion.

In cases where the fragment profile is identical to that produced by the control (pWR127 derived from a Vi⁺ strain), the Vi⁻ phenotype can be attributed to a mutation in *viaA*. This is based on the observation that retransformation of such plasmids into HB101 invariably resulted in production of Vi⁺strains, suggesting that the *viaB* function is intact. Genetic tests performed on the host bacteria invariably confirmed the presence of a *viaA* mutation. The *viaA* gene appears to be identical to the *rcsB* gene (Houng et al. 1991; see Stout and Gottesman 1990 for *rcsB*). Of the 95 independent Vi⁻ strains tested, 33 strains (35%) were found to carry a *viaA* mutation, while 49 strains (51%) were associated with an insertion of IS*1*. The rate of Vi⁻ formation due to deletion was quite high, at about 14%. The pWR127 DNAs isolated from eight of these independently derived deletion mutants all reacted positively with the IS*1* DNA probe in a DNA-DNA hybridization test (data not shown).

These revealed that there are about 10 insertion sites for IS*1* in pWR127 (Fig. 1, Table 2). As shown elsewhere (Ou et al. 1988; Rubin et al. 1985) the *viaB* region in pWR127 consists of two *Eco*RI fragments, EcoA (8.5 kb) and EcoB (9.5 kb), and all 10 insertion sites were clustered in EcoB, within a region located between 2.05 kb and 0.7 kb from the junction with the vector. Based on 49 samples analyzed, IS*1* inserted preferentially into four sites at positions 1.3, 1.5, 1.65, and 1.75 kb.

Stability of Vi⁺ phenotype in various strains

As shown earlier (Ou et al. 1988) and above, Vi⁺ HB101/pWR127 converts to Vi⁻ at a high frequency. A number of other Vi⁺ strains are known, some of which were artificially converted to Vi⁺ by mating with Vi⁺ donors or by transformation of Vi⁺ pWR127. The stability of the Vi expression state was qualitatively examined in nine Vi⁺ strains (Table 3), i.e., the ability to generate Vi⁻ strains was carefully explored. Two *C. freundii* strains, OU7177 and WR7004 (Ou et al. 1988), and *E. coli* K–12 OU3210 were readily converted from the Vi⁺ state to the Vi⁻ state. However, conversion could proceed in both directions, from Vi⁺ to Vi⁻ and vice versa, in OU7177 and WR7004 but only from Vi⁺ to Vi⁻ in OU3210. The *E. coli* C strain OU3327 was also shown to convert from Vi⁺ to Vi⁻, though at a very low frequency. The other strains tested, *S. typhi* OU6060 and *S. dublin* OU7178, exhibited stable expression of the Vi⁺ state, i.e., no conversion to Vi⁻ was observed.

To further investigate the stability of the Vi⁺ state in *S. typhimurium*, three *S. typhimurium* Vi⁻ strains were transformed with pWR127 and Vi⁺ colonies were isolated. All three strains that received pWR127 (Table 3) displayed a stable Vi⁺ phenotype, as assayed visually and serologically. In addition, the *Pst*I fragment profile was identical to that produced by Vi⁺ pWR127, indicating that no insertion events had taken place.

Table 2. IS1 insertion sites and frequencies

	Distance from the junction with pACKC1 (kb)										Total
	0.7	0.8	1.1	1.22	1.3	1.52	1.65	1.75	1.95	2.05	
No. of strains	1	1	3	3	8	8	7	11	2	5	49
%	2	2	6	6	16.3	16.3	14.3	23	4	10	99.9

Table 3. Bacterial strains, stability of the Vi antigen expression and the presence of IS1

	Strain	Species[a]	Relevant plasmid	Vi expression conversion[b]	Presence of IS1[c]
1	WR7004	C.f.	–	$Vi^+ \leftrightarrow Vi^-$	+
2	OU7177	C.f.	–	$Vi^+ \leftrightarrow Vi^-$	+
3	OU5140	S.t.	pWR127	Vi^+, stable	–
4	OU5146	S.t.	pWR127	Vi^+, stable	–
5	OU5316	S.t.	pWR127	Vi^+, stable	–
6	OU6060	*S. typhi*	–	Vi^+, stable	–
7	OU7178	*S. dublin*	–	Vi^+, stable	–
8	OU3210	E.c.K–12	pWR127	$Vi^+ \rightarrow Vi^-$	+
9	OU3327	E.c.C	–	$Vi^+ \rightarrow Vi^-$	+

[a] See footnote in Table 1

[b] ↔, reversible conversion; →, one-way conversion OU6060, OU7178, and OU3327 were from the WRAIR stock and their Vi^+ characteristic was originally transferred from C.f. Hfr WR7005 by mating

[c] +, present; –, absent

Distribution of IS1 in various strains

Our previous observations (Ou et al. 1988) had indicated that loss of the ability to produce Vi antigen is caused by IS*1* insertion. We, therefore, carried out DNA-DNA hybridization to determine the distribution of IS*1* in a variety of bacterial strains, including those described above. In some cases, isolated chromosomal DNA and in others, *in situ* colony hybridization was used for these tests. No differences between the two methods were observed. Table 3 lists only the results for Vi^+ strains. Not included in Table 3 are 3 laboratory strains and 7 clinical isolates of Vi^- *C. freundii*, all of which showed the presence of IS*1*. As shown in Table 3, all Vi^+ strains that generate Vi^- variants, such as WR7004, OU7177, OU3210, and OU3327 hybridized with the IS*1* probe.

Presence of IS1 induces insertion

Strain OU5140 (containing pWR127, see Table 3) has never been observed to produce Vi^- colonies. Our tests show that it does not contain IS*1* (Table 3). To determine the effect of the presence of IS*1* on the ability to form Vi^- strains, pRR134, a plasmid that contains IS*1* (Peterson et al. 1982) was introduced into OU5140 by transformation. The Vi^+ transformants were incubated in broth, streaked on nutrient plates and checked for the presence of Vi^- colonies. Vi^- colonies were generated in considerable numbers. To determine whether or not the insertion of an IS*1* element is associated with the appearance of these Vi^- colonies, plasmid DNAs from these Vi^- strains were isolated, purified, and digested with restriction endonucleases *Eco*RI and *Pst*I. The enzyme-treated plasmids were then electrophoresed in a 1% agarose slab gel. The two plasmids, pWR127 and pRR134, could be distinguished readily, based on size differences and their distinct fragment profiles. The pWR127 derived from the Vi^+ strains of OU5140/pRR134 produced fragment profiles identical to that produced by pWR127 derived from Vi^+ OU3210, whereas a typical insertion profile was readily observed from those derived from Vi^- OU5140/pRR134 (data not shown). In addition, deletions in pWR127 were also seen.

Discussion

Reexamination of the locations of IS*1* insertions in Vi^- strains indicate that insertions occur at many sites but not in a random fashion (Table 2). Ten insertion sites, distributed within an area of approximately 1.3 kb, have been found so far, and the frequency of insertion at each site is not the same: insertions occur more frequently at four sites than at the others. The presence of many insertion sites may contribute to the high rate of conversion to Vi^- from Vi^+ (Ou et al. 1988). Why the insertion sites are confined to a region of about 1 kb is unknown. Since any insertion in this region results in Vi^- expression, the promoter and the genes that are transcribed early may be located in this region.

IS*1* is known to cause deletions (Reif and Saedler 1975), and furthermore, IS*1*-mediated deletion has been shown to begin from one end of the IS*1*, leaving the IS*1* in place (Ohtsubo and Ohtsubo 1978). Our hybridization tests on eight deletion DNAs showed the persistence of IS*1*, consistent with this finding. In our sample, we note that of 95 Vi^- pWR127 examined, 14% showed deletions. This is an extremely high frequency, since pWR127 must first pick up an IS*1* insertion (at a frequency of $2.6 \times 10^{-4} \times 0.51 = 1.33 \times 10^{-4}$ per bacterium per generation, see Ou et al. 1988). Thus, the 14% scored as deletions should also be regarded as having insertions, the rate of insertion then becomes 65% or 1.69×10^{-4} per bacterium per generation. An unusually high rate was also observed for the conversion of Vi^+ to Vi^- due to mutations in *viaA* (33 out of 95 Vi^- pWR127 or 35%). This is equivalent to 9.1×10^{-5} ($2.6 \times 10^{-4} \times 0.35$) per bacterium per generation, which is about 10^5-fold higher than the rate of spontaneous mutation. Since Vi^- is rarely seen in strains devoid of IS*1*, the high *viaA* mutation rate seems to be correlated with the presence of IS*1*. In the present case, we detected *viaA* mutations only in the presence of pWR127, with which detection was relatively easy. The reason for such a high mutation rate in *viaA* is still unknown.

Of the 9 Vi^+ strains that were examined for the stability of Vi expression, four strains, WR7004, OU7177, OU3210, and OU3327, produced Vi^- strains, but the rest were very stable, and were never seen to generate Vi^- strains. The requirement for IS*1* in the Vi^- production of strains is consistent with the observed ability of OU3210 and OU3327 strains, both of which are found to contain IS*1* (Table 3) to segregate Vi^- variants. However, Nyman et al. (1981) have reported that *C. freundii*, at least the strain they checked, does not harbor IS*1*. This observation conflicts with our finding and the notion that IS*1* is required for the generation of Vi^- strains in WR7004 and OU7177, both are *C. freundii* strains. The

results of DNA-DNA hybridization tests (Table 3) clearly revealed that all stable Vi^+ strains contain no IS*1*, and that all Vi^--producing strains harbor IS*1*, consistent with the notion that IS*1* is necessary for Vi^- formation. Note that all *C. freundii* strains we tested, regardless of the source, and including the laboratory strain WR7004, contain IS*1*, indicating that the *C. freundii* strain examined by Nyman et al. (1981) may have been an exceptional one that happened to be devoid of IS*1*. This is not unusual because WR7022, a *recA* Vi^- mutant derived from WR7004, has also lost IS*1* (data not shown). Furthermore, when WR7022 harbors Vi^+ pWR127, its Vi^+ state is very stable; it has not been observed to generate Vi^- strains. Our tests for the presence of IS*1* in various strains, however, confirmed another result of Nyman et al. (1981): *S. typhimurium* does not contain IS*1*. Consequently, we expected, and found, that all three *S. typhimurium* strains harboring a Vi^+ pWR127 stably remain in the Vi^+ state.

On the other hand, if IS*1* elements are introduced into these stable Vi^+ strains that do not contain IS*1*, one would predict the appearance of Vi^- strains. This is indeed the case when pRR134, which contains IS*1*, is transferred into OU5140, a *S. typhimurium* strain containing a Vi^+ pWR127: Vi^- strains were readily produced.

The presence of IS*1* is now clearly shown to be necessary for the production of Vi^- strains in all the strains tested except for *C. freundii*. This mode of inhibition of gene expression is also found to be the cause of loss of virulence in *Shigella flexneri* (Mills et al. 1992). Vi expression in *C. freundii*, unlike other strains examined so far, is readily reversible, and its precise mechanism is still unknown. In conjunction with the observation of IS*1* insertions in other strains, the finding that *C. freundii* appears to contain IS*1* opens up the possibility that this element is also involved in the regulation of Vi expression in this species.

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